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Characterization of a 46 kDa Insect Chitinase from Transgenic Tobacco

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A 46 kDa *Manduca sexta* (tobacco hornworm) chitinase was isolated from leaves of transgenic tobacco plants containing a recombinant insect chitinase cDNA, characterized, and tested for insecticidal activity. The enzyme was purified by ammonium sulfate fractionation, Q-Sepharose anion-exchange chromatography and mono-S cation-exchange chromatography. Although the gene for the chitinase encoded the 85 kDa full-length chitinase as previously reported by Kramer *et al.* [*Insect Biochem. Molec. Biol.* 23, 691–701 (1993)], the enzyme is produced in tobacco as a 46 kDa protein that is approximately four-fold less active than the 85 kDa chitinase. The N-terminal amino acid sequence of the 46 kDa chitinase is identical to that of the 85 kDa chitinase. The former enzyme is not glycosylated, whereas the latter contains approximately 25% carbohydrate. The pH and temperature optima of the 46 kDa chitinaseare similar to those of the 85 kDa chitinase. The former enzyme is more basic than the latter. The 46 kDa chitinase likely consists of the N-terminal catalytic domain of the 85 kDa chitinase and lacks the C-terminal domain that contains several potential sites for glycosylation. The 46 kDa chitinase is expressed in a number of plant organs, including leaves, flowers, stems and roots. Enzyme levels are higher in leaves and flowers than in stems and roots, and leaves from the middle portion of the plant have more chitinase than leaves from the top and bottom portions. Little or no enzyme is secreted outside of the plant cells because it remains in the intracellular space, even though its transit sequence is processed. When fed at a 2% dietary level, the 46 kDa chitinase caused 100% larval mortality of the merchant grain beetle, *Oryzaephilis mercator*. The results of this study support the hypothesis that insect chitinase is a biopesticidal protein for insect pests feeding on insect chitinase gene-containing transgenic plants. Published by Elsevier Science Ltd

Chitinase Tobacco Transformation Gene *Manduca sexta* Tobacco hornworm Bioassay *Oryzaephilus mercator* Merchant grain beetle

INTRODUCTION

For several decades, synthetic chemical insecticides have been some of the most powerful tools available for use in pest management. However, reliance on these insecticides as the primary control method has created serious problems, and novel and safer methods for control of insect pests are required. The enhancement of genetic resistance in plants to damage by insect predation is promising, but is hampered by a shortage of useful genes for this purpose. Two types of genes that are being developed to enhance resistance in plants to insects are genes for insecticidal δ-endotoxins from the bacterium, *Bacillus thuringiensis* (Bt) (Hoffman *et al.*, 1992), and proteinase inhibitors (PI) (Gatehouse *et al.*, 1992). Bt parasporal proteins or modified versions have a disruptive effect on insect gut cell membranes, resulting in paralysis and death of the host insect. PIs are targeted to specific types of proteinases that occur in the digestive tracts of insects.

Another potential insect control protein is the developmentally regulated insect molting enzyme chitinase, which degrades chitin in the gut peritrophic membrane and exoskeleton (Flach *et al.*, 1992). A long-term goal in our laboratory is to control pest insects by manipulating chitin metabolism using the insect chitinolytic enzymes (Kramer *et al.*, 1996). When an insect molts,

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two enzymes, chitinase and β-*N*-acetylglucosaminidase, are expressed in a hormonally regulated manner over a relatively short period of the larval stadium (Fukamizo and Kramer, 1987). If an insect is exposed to these enzymes outside of this time frame, the chitin-containing peritrophic membrane and cuticle might be damaged, leading to growth inhibition and mortality.

Both types of chitinolytic enzymes from *M. sexta* have been purified and characterized previously, and antibodies are available (Kramer and Koga, 1986). Using these antibodies, cDNA clones encoding chitinase and β-*N*-acetylglucosaminidase have been isolated and sequenced (Kramer *et al.*, 1993; Zen *et al.*, 1996). The cDNA clone encoding chitinase is 2452 nucleotides long with an open reading frame of 1662 nucleotides, which encodes a protein of 554 amino acids with a theoretical molecular mass of 62 kDa. The native chitinase and the recombinant *M. sexta* chitinase from a baculovirus-insect cell expression system are identical in size (85 kDa). Both are glycoproteins with carbohydrate accounting for apparently 25% of the mass (Gopalakrishnan *et al.*, 1995).

When the full-length insect chitinase gene was introduced into tobacco by *Agrobacterium*-mediated transformation, two enzymatically active and immunoreactive proteins with sizes of 46 and 85 kDa were detected in leaf extracts with the chitinase antibody (Ding, 1995). The smaller protein was the predominant form in western blot analysis. When transgenic tobacco plants with the chitinase gene were used in a feeding study with *Heliothis virescens* (tobacco budworm) larvae, the extent of leaf feeding was substantially lower than that of control plants. In addition, the weight gain of larvae reared on plants expressing the insect chitinase was significantly less than that of larvae grown on control plants.

Until now, it has not been determined what structural modifications have occurred in the 46 kDa protein or whether growth inhibition was a direct result of the enzyme activity. In this paper, a method for the purification of the 46 kDa chitinase from transgenic tobacco is described. Some of the properties of this truncated enzyme expressed in tobacco are compared with those of the full-length enzyme prepared from an insect cell line infected with a baculovirus containing the recombinant *M. sexta* chitinase cDNA (Gopalakrishnan *et al.*, 1995). We demonstrate that both glycosylation and the C-terminal portion of *M. sexta* chitinase are not essential for either enzymatic or insecticidal activity.

MATERIALS AND METHODS

Materials

The materials used for enzyme purification and their sources are as follows: Q-Sepharose fast flow and mono-S columns, Pharmacia; Immobilon-P transfer membranes for western blotting, Millipore; nitrocellulose membranes for dot blotting, MSI Micron Separations Inc.; Centricon 3 and Centriprep 3, Amicon; and phenylthiourea (PTU), phenylmethylsulfonylfluoride (PMSF), leupeptin, pepstatin and Fluorescent Brightener 28, Sigma.

The 85 kDa chitinase was prepared using a baculovirus-insect cell line for gene expression (Gopalakrishnan *et al.*, 1995) and purified to homogeneity (unpublished data). The substrate for the solution activity assay, CM (carboxymethyl)-Chitin-RBV (remazol brilliant violet), was from Loewe Biochemica GmbH (Nordring bei München, Germany). The substrate for the activity overlay gel, glycolchitin, was prepared according to Koga and Kramer (1983).

Tobacco transformation

The *Agrobacterium*-mediated transformation of tobacco leaf disks with a plasmid containing *M. sexta* chitinase cDNA was done using the procedure of Horsch *et al.* (1985). The 1.8 kb Eco R1 fragment from the chitinase cDNA clone 201 (Kramer *et al.*, 1993) was inserted into *Agrobacterium tumefaciens* transfer vector pMON410A. In this construct, pMON410A.chi, the chitinase gene is under control of the cauliflower mosaic virus 35S promoter. After regeneration, the plantlets were transferred to the greenhouse and allowed to set seed. *M. sexta* chitinase-positive plants were identified by western blot analysis. Seeds from self-fertilized chitinase-positive plants were germinated and grown in the greenhouse to maturity. Middle leaves from these plants were used for chitinase purification.

Purification of the 46 kDa chitinase

Leaves (800 g) were washed in deionized water, and excess moisture was removed using paper towels. They were homogenized for 5 min using a Waring blender at speed 8 in 10 mM Tris-HCl pH 7.5 containing 0.1 mM PTU, 100 μg/ml PMSF, 1 μg/ml pepstatin and 0.5 μg/ml leupeptin at 4°C. Four ml of the extraction buffer per gram of leaves were used. Before extraction, the buffer was cooled to 4°C. The crude extract was filtered through two layers of cheesecloth and centrifuged at 23 500 ***g*** for 30 min at 4°C. The supernatant was subjected to ammonium sulfate fractionation. Precipitates from 0–50% and 50–75% saturations were collected, dissolved in extraction buffer, and then dialyzed against a 100-fold volume of the buffer for 24 h with two additional changes of buffer.

Q-Sepharose fast flow anion-exchange medium (Pharmacia) was washed as recommended (Pharmacia Manual), placed in a 1.5×20 cm column with a total bed volume of 30 ml, and equilibrated with 50 mM Tris-HCl buffer pH 7.5. After the sample was applied to the column, 2 ml of 50 mM Tris pH 7.5 was added. Then the column was subjected to a gradient of 10 mM NaCl/min using 50 mM Tris pH 7.5 with 1 M NaCl as the second mobile phase. Fractions of 1 ml were collected at a flow rate of 1 ml/min and assayed for immunoreactive chitinase-like proteins by spotting 10–100 μl aliquots on a nitrocellulose membrane using the minifold blotter (Schleicher and Schuell Inc.). Then the membrane

was incubated with the *M. sexta* chitinase antibody to detect fractions containing chitinase. Peak fractions containing chitinase were pooled and dialyzed against 10 mM sodium acetate at pH 5.

A prepacked Mono-S HR 5/5 (1 ml) cation-exchange column (Pharmacia Biotech) was utilized for the final chromatography using a Beckman System Gold HPLC. The low salt buffer was 50 mM sodium acetate pH 5, and the high salt buffer was 50 mM sodium acetate pH 5 containing 1 M NaCl. The flow rate was 1 ml/min with a gradient of 10 mM NaCl/min. Chitinase was eluted at 0.4 M NaCl. Aliquots of the fractions containing chitinase were examined by SDS-PAGE followed by silver staining (Nesterenko *et al.*, 1994). The fractions containing the 46 kDa chitinase were desalted by ultrafiltration using a Centricon filter (molecular weight cut off 3K, Amicon).

Western blotting

Western blotting was done according to Winston *et al.* (1987). Samples were run on 10% SDS-polyacrylamide gels. Immediately after electrophoresis, gels were blotted onto Immobilon-P transfer membranes (Millipore) using a Milliblot SDE apparatus (Millipore) according to the manufacturer's instructions. The membranes were blocked with 2.5% gelatin for 1 h. They were then treated with the first antibody overnight. The *Manduca* polyclonal chitinase antibody was raised in rabbits against the 85 kDa chitinase purified from molting fluid (Koga *et al.*, 1983). The protein bands cross-reacting with the chitinase antibody were visualized using blotting grade horseradish peroxidase color-development reagent (4-chloro-1-naphthol) from Bio-Rad as described by Gopalakrishnan *et al.* (1995).

Dot blotting

Protein solutions were applied directly to the nitrocellulose membrane using the minifold dot blotter, and the membrane was dried at 65°C in an oven. The membrane was blocked, incubated with first and second antibodies, and developed as was done for western blotting.

Expression, tissue localization and subcellular localization of the 46 kDa M. sexta *chitinase in transgenic tobacco leaves*

Transgenic tobacco plants expressing chitinase were grown in the greenhouse until the plants had at least 20 leaves. Seven leaf discs of diameter 0.5 cm were punched out from every third leaf and placed in a microcentrifuge tube. The discs were ground in liquid nitrogen to a powder and extracted with 0.5 ml of phosphate buffer containing 1 μg/ml pepstatin, 0.5 μg/ml leupeptin and 100 μg/ml PMSF. The samples were centrifuged at 12 000 ***g*** for 10 min, and the supernatants were collected. Total protein concentration of each sample was determined by the bicinchoninic acid (BCA) microtiter plate assay (Pierce). Equal amounts of total protein (250 μg) were subjected to SDS-PAGE followed by western blotting analysis.

Leaves, stems, roots and flowers from transgenic tobacco plants were collected, ground in liquid nitrogen and extracted in potassium phosphate buffer pH 6.8 containing 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, and 100 μg/ml PMSF. Total protein concentrations of the supernatants were determined by the BCA protein assay, and equal amounts of protein were subjected to SDS-PAGE followed by western blotting analysis.

Intercellular fluid was extracted following the method of Mauch *et al.* (1984). Middle leaves from three transgenic tobacco plants were collected. Leaves were cut into halves and washed extensively in water. They then were vacuum infiltrated for 10 min in 0.05 M phosphate buffer (pH 7.5) containing 5 mM ethylenediaminetetraacetic acid (EDTA), 0.01 M β-mercaptoethanol, 100 μg/ml PMSF, 1 μg/ml pepstatin, and 0.5 μg/ml leupeptin. Leaf strips were blotted dry and put into a 10 ml syringe, which was placed on top of a sterile microcentrifuge tube and then centrifuged at 800 ***g*** for 10 min. The intercellular fluid at the bottom of the tube was collected. Leaf pieces from the syringe were frozen in liquid nitrogen and extracted with the same potassium phosphate buffer as above. The entire intercellular fluid and the intracellular extracts from the leaves (approximately 500 μg total protein) were subjected to TCA precipitation and western blot analysis as described above. For the TCA precipitation, aliquots containing the desired amount of protein were mixed with an equal volume of 20% TCA and kept at 4°C for 20 min. After centrifugation at 10 000 ***g***, the precipitates were washed three times with acetone and air-dried before dissolving in sample buffer for gel electrophoresis.

Native polyacrylamide gels and activity assay

Samples were subjected to electrophoresis in a 10% native polyacrylamide minigel at pH 8.8 (Blackshear, 1984). After electrophoresis, the gel was overlaid with a 7.5% polyacrylamide gel containing 0.01% glycol chitin as substrate and incubated at 37°C for 5 h following the procedure of Trudel and Asselin (1989) with minor modifications. Chitinase bands were detected by the absence of staining with Fluorescent Brightener 28 (Sigma) when viewed under ultraviolet light.

Chitinase activity assay

To obtain a more quantitative measure of chitinase activity, a colorimetric assay with CM-Chitin-RBV as the substrate was utilized. Samples were diluted with water to 0.4 ml and incubated at 37°C with 0.2 ml of 0.2 M phosphate-citrate buffer pH 7.5 and 0.2 ml of CM-Chitin-RBV (2 mg/ml) for 1 or 2 h. The reaction was stopped by the addition of 0.2 ml of 2 N HCl, and samples were cooled on ice for 15 min. After the samples were centrifuged at 12 000 ***g*** for 5 min, the absorbance at 550 nm of the supernatant was determined using a UV Max Kinetic Microplate Reader (Molecular Device).

N-Terminal sequencing

Proteins were subjected to SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and stained with Ponceau S. The 46 kDa chitinase band was cut out and subjected to automated Edman degradation using an Applied Biosystems Sequencer (Matsudaira, 1987; Tempst and Riviere, 1989) at the Biotechnology Microchemical Core Facility, Kansas State University, Manhattan, KS.

Carbohydrate analysis

Proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. The protein band corresponding to chitinase was cut out from the gel, and its carbohydrate composition was determined at the Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO. Samples were hydrolyzed to liberate carbohydrate moieties, and sugars were reduced and derivatized to alditol or hexosaminitol acetates, which were separated by gas–liquid chromatography and quantified by mass spectrometry (Mawhinney *et al.*, 1980; Mawhinney, 1986; Tilley *et al.*, 1993).

Molecular weight determination

The molecular mass was determined by electrospray and laser desorption mass spectrometry at the Protein and Carbohydrate Structure Facility at the University of Michigan. Electrospray ionization mass spectra were obtained using a VG/Fisons "platform" electrospray ionization single quadrupole mass spectrometer. Samples were delivered to the source in a 20 μl injection loop at 20 μl/min in 4% acetic acid/50% acetonitrile.

For laser desorption mass spectrometry, the dried proteins were redissolved into 100 ml 0.1% TFA. An aliquot of protein (~10 picomoles in 1 μl) was mixed with or without ~0.5 picomole of horse heart myoglobin as an internal standard and 1 μl of saturated matrix solution (50 mM sinapinic acid). The mass spectrometer was a Lasertec Research Vestec-2000 laser desorption linear time-of-flight mass spectrometer (Houston, TX) equipped with a 1.2 m flight tube. It was operated at a 23 kV ion accelerating voltage and 3 kV multiplier voltage using a 337 nm VSL-337 ND nitrogen laser (Laser Science, Inc.) with a 3 ns pulse width. Spectra were summed from 10 to 15 laser shots.

Isoelectric points of 46 and 85 kDa chitinases

Two-dimensional isoelectric focusing/SDS-PAGE was performed according to the method of O'Farrell (1975) using tropomyosin as an internal standard (Kendrick Laboratories, Inc., Madison, WI). The pH gradient was determined using a surface microelectrode.

Calculation of theoretical isoelectric points of 46 and 85 kDa recombinant insect chitinases

The theoretical isoelectric points (pIs) of the chitinases were calculated by IBI Pustell Sequence Analysis Program, Ver. 2.04, from the amino acid composition deduced from the predicted amino acid sequence of the mature proteins.

pH profile

Chitinase activity assays at different pH values were done using 0.5 μg/ml of chitinase at 37°C for 2 h. Buffer compositions were citrate–citric acid for the pH range 4–5.5, sodium phosphate between pH 6.0 and 8, Tris-HCl at pH 8.5, glycine-NaOH at pH 9 and 10, and sodium phosphate at pH 11. This experiment was replicated three times.

Temperature profile

The activity of chitinase as a function of temperature was measured by first incubating the CM-Chitin-RBV substrate at pH 8 in phosphate buffer for 1 h. Then enzyme (0.5 μg/ml) was added, and the mixtures were incubated in triplicate for 2 h at various temperatures, after which the absorbance at 550 nm was recorded.

Insect bioassay

Raw wheat germ (29.2 mg) was mixed with a solution of recombinant 46 kDa insect chitinase (0.596 mg/45 μl). Raw wheat germ (30 mg) was mixed with water (45 μl) as a control. After freeze drying, the diets were ground in a mortar with a pestle. From 2.8 to 3.2 of mg diet was transferred to 0.5 ml microcentrifuge tubes and equilibrated at 28°C and 70–75% relative humidity. A single egg (0–24 h after oviposition) of the merchant grain beetle, *Oryzaphilus mercator*, was added to each individual vial. The vials were capped, and two holes were made in the lids with a no.1 insect pin to allow air exchange. Mortality was monitored every third day, and larvae were weighed after 15 days.

RESULTS

Purification of 46 kDa recombinant insect chitinase from transgenic tobacco

The 46 kDa recombinant insect chitinase was purified from crude extracts of transgenic tobacco leaves by ammonium sulfate fractionation, Q-Sepharose anion exchange chromatography and mono-S cation-exchange chromatography. On the basis of immunoreactivity, most of the *M. sexta* chitinase was in the pellet obtained from the 50–75% ammonium sulfate fraction (data not shown). Therefore, this fraction was used for further purification. Upon Q-Sepharose anion-exchange chromatography, the recombinant enzyme was eluted at 70–90 mM NaCl and was well separated from the peaks of plant chitinase activity [Fig. 1(A)]. The Q-Sepharose fraction containing both chitinase activity and immunoreactivity (fractions 6–10) with *M. sexta* chitinase antibody next was subjected to chromatography using a Mono-S cation-exchange column at pH 5. The enzyme eluted at 400 mM NaCl [Fig. 1(B)]. SDS-PAGE analysis revealed

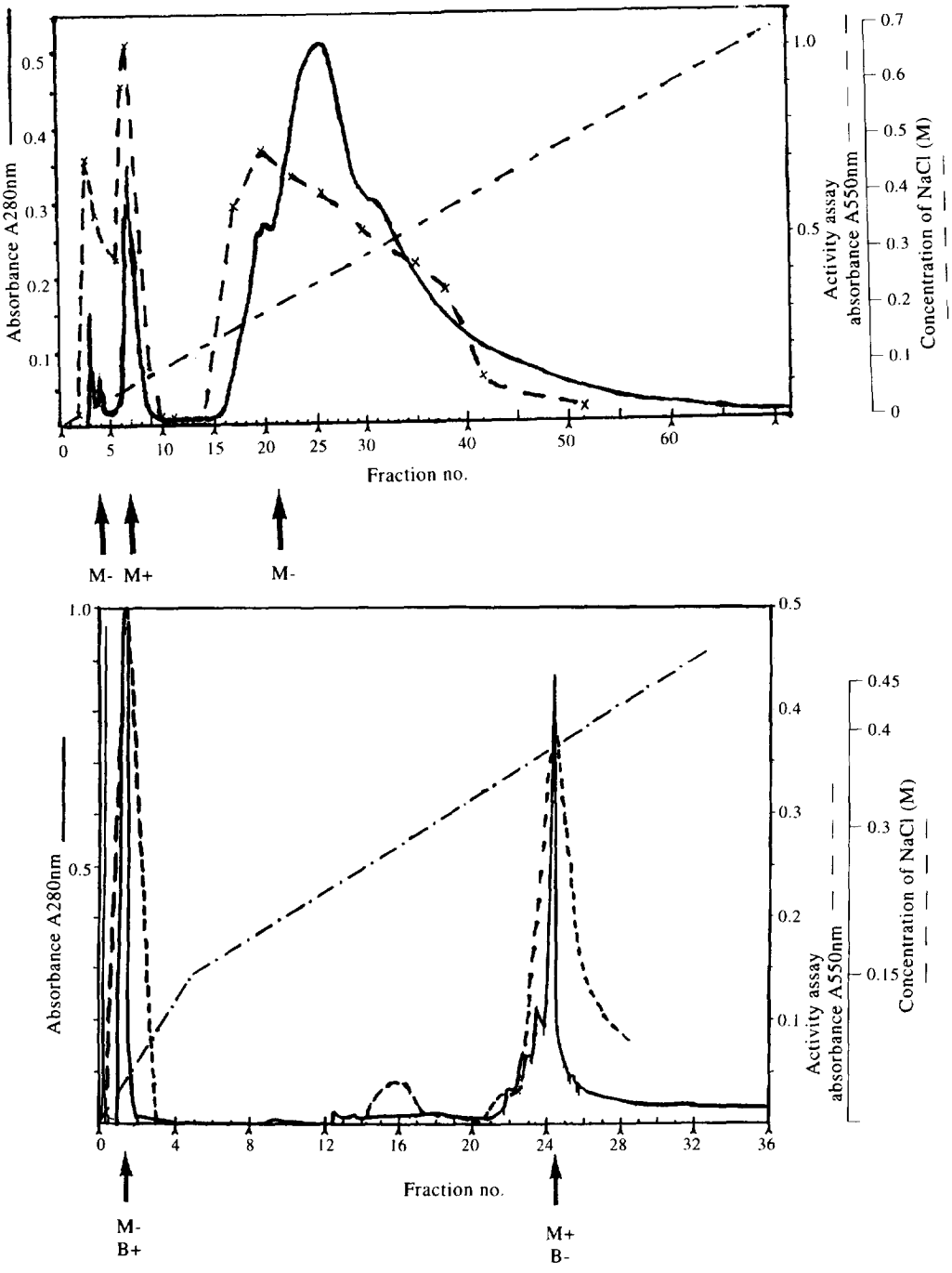

FIGURE 1. (A: top) Anion-exchange chromatography on Q-Sepharose of proteins from transgenic tobacco plants. Proteins from the 50–75% ammonium sulfate fraction were dissolved in 10 mM Tris pH 7.5 and dialyzed against the same buffer. Then the mixture was loaded onto a 1.5×20 cm Q-Sepharose column, and the proteins were eluted with a linear NaCl gradient at 4°C. Chitinase activity was assayed with CM-Chitin-RBV, as substrate as described in the Materials and Methods section. Absorbance at 280 nm (protein concentration), ———; absorbance at 550 nm (chitinase activity assay), – – –; and concentration of NaCl (M), —·—. The fractions from each activity peak were pooled and subjected to dot blot analysis using the antibody against *M. sexta* chitinase. M+, immunoreactive; M-, not immunoreactive. (B: bottom) Cation-exchange chromatography on a mono-S column of insect chitinase from the Q-Sepharose chromatography. The fractions containing insect chitinase from the Q-Sepharose chromatography were pooled and ultrafiltered against 50 mM sodium acetate using a Centricon 3 filter (Amicon). Then the protein mixture was passed over a prepacked mono-S HR 5/5 column (Pharmacia Biotech). The enzyme was eluted with a NaCl gradient: 0–5 min, 30 mM NaCl/min; 5–36 min, 10 mM NaCl/min. Enzyme activity was assayed by using CM-Chitin-RBV as substrate as described in the Materials and Methods section. Absorbance 280 nm, ———; activity assay, absorbance at 550 nm, – – –; and concentration of NaCl (M), —·—. The 46 kDa insect chitinase in fraction no.24 and the fractions from the first activity peak (nos. 1–3) were subjected to dot blot analysis using the antibody against *M. sexta* chitinase or bean chitinase. M+, immunoreactive with *M. sexta* chitinase antibody; M-, not immunoreactive with *M. sexta* chitinase antibody; B+, immunoreactive with bean chitinase antibody; B-, not immunoreactive with bean chitinase antibody. There is no immunological cross-reactivity between the *Manduca* chitinase and the bean chitinase antibodies (unpublished data).

that the purity of the chitinase was >95% and that its apparent molecular mass was approximately 46 kDa (Fig. 2). This preparation was used for subsequent experiments. The recombinant insect chitinase contributed approximately 8% and the endogenous tobacco chitinases 92% of the total activity in the crude extract.

Chitinase expression in different tissues

A preliminary western blot analysis comparing the levels of immunoreactive chitinase in the top, middle and bottom leaves of relatively young transgenic tobacco plants showed that the level of chitinase was higher in older leaves (bottom) compared to younger leaves (top). In order to confirm this observation and to determine whether chitinase expression follows a similar pattern in mature plants, three large plants were chosen from which every third leaf was collected for a total of seven or more leaves from each plant. The leaves beneath leaf 4 (counting from bottom to top), which were yellow, and the leaves above leaf 22, which were very small, were not used. The proteins were extracted, and the chitinase levels were analyzed by western blotting. Chitinase expression was highest in leaves from the middle portion of the plant [leaves 7–16; Fig. 3(A)]. For subsequent experiments, the 46 kDa chitinase was extracted from leaves from the middle portions of the transgenic plants.

To determine the tissue localization of expression of the 46 kDa chitinase in transgenic tobacco plants, protein extracts from roots, stems, leaves and flowers were assayed for *M. sexta* chitinase immunoactivity using western blotting. The level in the flowers was approximately half that present in the leaves, whereas chitinase levels in the stems and roots were even lower [Fig. 3(B)].

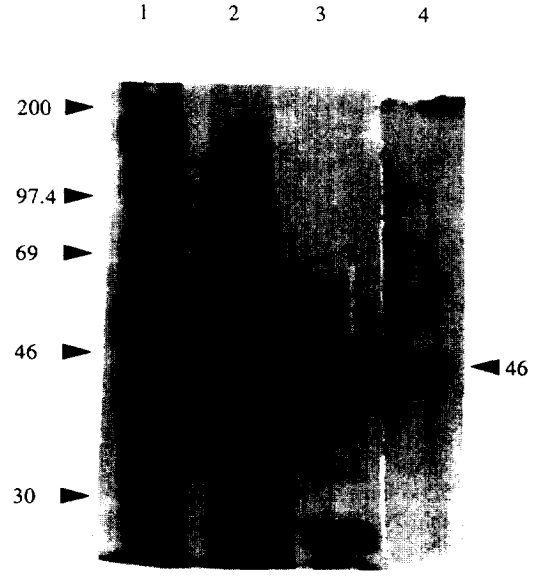

FIGURE 2. SDS-PAGE analysis followed by silver staining of fractions obtained from the purification of insect chitinase. (1) crude extract (20 μg); (2) 50–75% ammonium sulfate fraction (30 μg); (3) pooled fractions 5–10 that contain insect chitinase from Q-Sepharose column (5 μg); (4) fraction no.24 from mono-S column (1 μg).

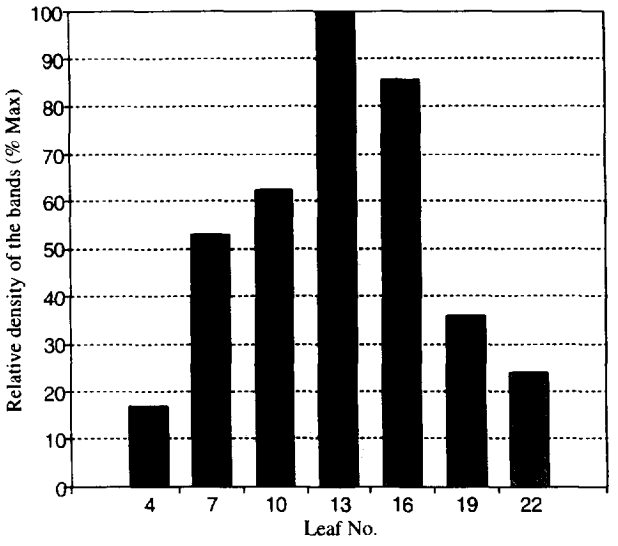

FIGURE 3. (A) Densitometric analysis of the western blot of 46 kDa insect chitinase levels in leaves of different ages from transgenic tobacco. The leaves are numbered by location from the bottom to the top of the plant. Leaves 1–3 were yellow, and those higher than leaf 22 were too small for analysis. Two hundred micrograms of total protein were loaded in each lane and analyzed for immunoreactive material as outlined in the Materials and Methods section.

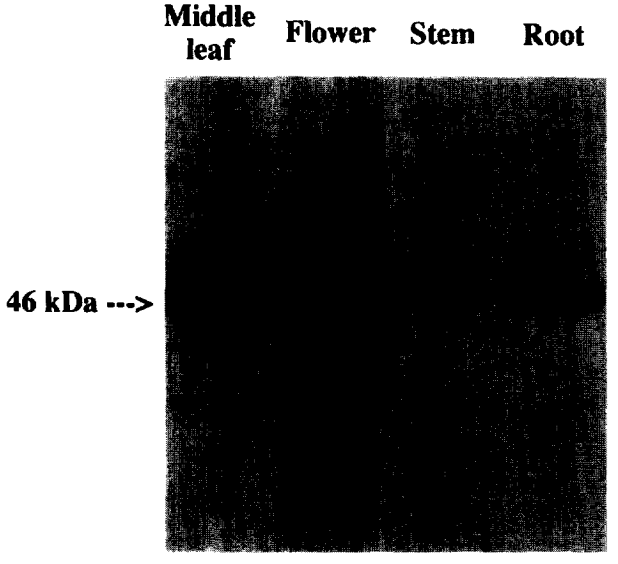

FIGURE 3. (B) Expression of 46 kDa insect chitinase in different tissues of transgenic tobacco plants. Two hundred micrograms of total protein from extracts of indicated tissues were loaded in each lane and subjected to western blot analysis.

Subcellular localization of the 46 kDa chitinase

To determine the subcellular localization of the 46 kDa chitinase, intercellular fluid and cell extracts were prepared from three leaves and subjected to western blot analysis. No 46 kDa chitinase was present in the intercellular fluid, whereas the enzyme was in the extracts of cells after removal of apoplastic fluid [Fig. 3(C)]. The recombinant chitinase remained in an intracellular location in the leaves and little, if any,was secreted outside of the cells.

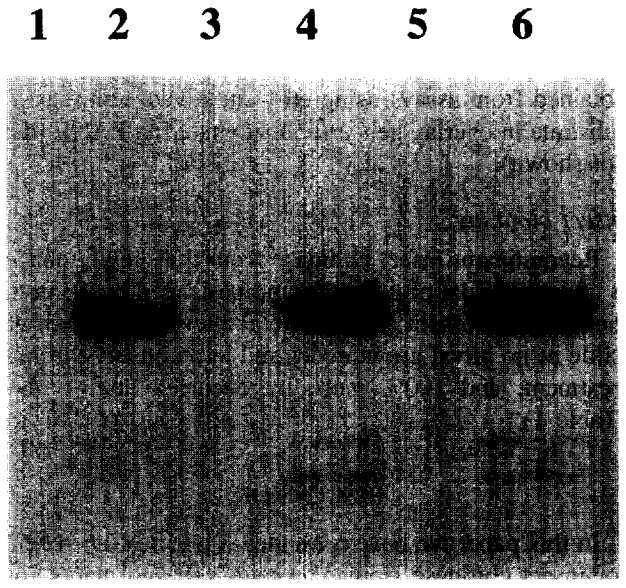

FIGURE 3. (C) Subcellular localization of 46 kDa chitinase in transgenic tobacco plants. Three leaves from the middle of the plant were processed as described in the Materials and Methods section to obtain the apoplastic fluid and cell extracts, which were analyzed as in Fig. 3(B) 1, 3 and 5= poplastic fluid, and 2, 4 and 6=extracts of leaves after removal of apoplastic fluid.

N-Terminal amino acid sequence analysis

The N-terminal amino acid sequence of the 85 kDa mature chitinase from *Manduca* molting fluid is DSRA-RIV (Kramer *et al.*, 1993; Gopalakrishnan *et al.*, 1995). In order to determine whether the smaller form of recombinant insect chitinase from transgenic tobacco has the same N-terminus, the 46 kDa chitinase was subjected to automated N-terminal sequence analysis. The N-terminal sequence was identical to that of the native enzyme, DSRARIV. The 46 kDa recombinant insect chitinase appears to be a truncated product of the full-length chitinase with the C-terminal portion of the protein absent.

Carbohydrate analysis

The mature *M. sexta* chitinase from molting fluid is a glycoprotein and carbohydrate content accounts for approximately 27% of the mass (Gopalakrishnan *et al.*, 1995). In contrast, the 46 kDa chitinase from transgenic plants contained little or no carbohydrate (data not shown). The C-terminal region, presumed to be missing in the 46 kDa chitinase, contained several possible N-glycosylation and O-glycosylation sites based on the sequence (Kramer *et al.*, 1993). Perhaps only the glycosylation sites in the C-terminal end, which are absent in the nonglycosylated 46 kDa chitinase, serve as acceptor sites for protein glycosylation. Therefore, the 46 kDa insect chitinase produced by transgenic tobacco was smaller than the 85 kDa native chitinase because the former was not a glycoprotein and the C-terminal portion of the amino acid sequence was missing.

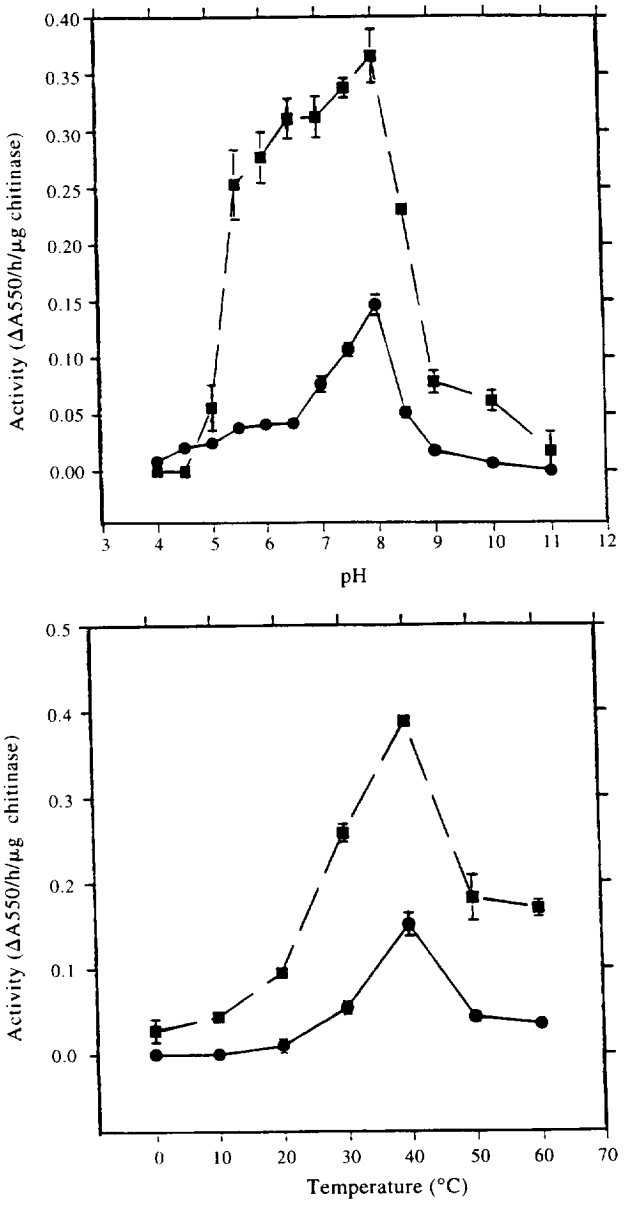

FIGURE 4. (A) pH-activity profiles of insect chitinases. The activities as a function of pH of purified 46 kDa recombinant insect chitinase from transgenic tobacco and 85 kDa insect chitinase purified from culture medium of insect cell line (Hi-5) infected with a baculovirus containing *M. sexta* chitinase cDNA (Gopalakrishnan *et al.*, 1995) were determined as outlined in the Materials and Methods section. Data are presented as $\Delta A550/h/\mu g$ protein. Mean values from three determinations are plotted. Error bars show standard deviations. 46 kDa chitinase, ● and 85 kDa chitinase, ■. (B) Temperature–activity profiles of 46 and 85 kDa insect chitinases. Data are presented as $\Delta A550/h/\mu g$ protein. Mean values from three determinations are plotted. Error bars show standard deviations. 46 kDa chitinase, ● and 85 kDa chitinase, ■.

pH and temperature optima

Enzymatic assays using CM-Chitin-RBV were conducted at different pH values using the 46 and 85 kDa recombinant chitinases. The smaller chitinase was most active at pH 8 and the activity decreased rather precipitously when the pH was increased or decreased [Fig. 4(A)]. The enzyme was essentially inactive at pH<5 and

at pH>8.5. The larger chitinase, on the other hand, was active over a broader pH range, but its maximum activity occurred at pH 8. The 85 kDa enzyme was inactive at pH<5 and pH>10. Therefore, the pH activity profiles for the two enzymes were more similar on the alkaline side of the pH optimum and slightly dissimilar on the acidic side.

The two chitinases were assayed at the optimum pH 8 over the temperature range of 0–60°C at intervals of 10°C. For both chitinases, the dependence of enzymatic activity on temperature was quite similar with maximum activities occurring at 40°C [Fig. 4(B)]. Activity levels fell rapidly when the temperature was below or above 40°C.

Molecular mass of the recombinant insect chitinase

The molecular mass of the *M. sexta* chitinase from transgenic tobacco was determined by electrospray mass spectrometry to be 46 099±16 Da. Results from laser desorption mass spectrometry revealed that three proteins of masses 45 914, 46 081, and 46 190 Da likely are present in the preparation. All of these values are consistent with the size of 46 kDa estimated by SDS-PAGE.

From the N-terminal sequences of the *M. sexta* 85 and 46 kDa chitinases and the molecular masses of the latter, a truncation site for the 46 kDa chitinase can be proposed. The *M. sexta* chitinase expressed in tobacco plants appears to be truncated after 407 amino acids (theoretical MW=46 085 Da), such that the 46 kDa recombinant truncated chitinase consists of the first 407 N-terminal amino acid residues of the mature 85 kDa enzyme (after removal of the 19 amino acid-long leader peptide). The 46 kDa enzyme thus contains the catalytic domain of the 85 kDa chitinase, but lacks the glycosylation sites of the larger enzyme.

Isoelectric points of insect chitinases

Upon two-dimensional gel analysis, purified 46 and 85 kDa chitinases were resolved into multiple spots, indicating the presence of multiple forms of chitinase that have nearly the same molecular mass, but slightly different charges (data not shown). The pI values of the three major forms of the 46 kDa chitinase are pH 7.1, 7.0 and 6.9, whereas those of the three major forms of the 85 kDa chitinase are pH 6.1, 6.0 and 5.9. Thus, the truncated chitinase is slightly more basic than the full-length chitinase. The predicted pI values of the 46 and 85 kDa chitinases are approximately pH 5.8 and 5.1, respectively. The reason for the differences between predicted and measured pI values as well as the occurrence of multiple forms is unknown.

Enzymatic activities

Using the hydrolysis of CM-chitin-RBV as the assay method, the specific activity of the 46 kDa chitinase at 40°C and pH 8 was $6.9\pm0.1\times10^9$ ΔA550/h/mole, whereas that of the 85 kDa enzyme was $2.9\pm0.1\times10^{10}$ ΔA550/h/mole. The specific activity of the full-length chitinase is 4.2 times greater than that of the smaller enzyme. The results agree with semiquantitative data obtained from assays using ethylene glycol chitin as the substrate in overlay gels after nondenaturing PAGE (data not shown).

Insect bioassay

Recombinant insect chitinase from transgenic tobacco is an insecticidal protein when administered orally in the diet of the merchant grain beetle at a level of 2% (wt/wt). Little or no larval growth occurred in the insect chitinase treatment, and 100% mortality occurred after 6 days (Table 1).

DISCUSSION

In this paper we have presented a purification scheme for insect chitinase from transgenic tobacco plants containing a *Manduca sexta* chitinase cDNA. Plants expressing the chitinase gene produce a 46 kDa protein that reacts with an antibody against the insect chitinase and possesses chitinolytic activity. The size of this protein is substantially smaller than that of the mature proteins found either in *M. sexta* molting fluid or the culture medium of insect cells infected with a baculovirus expressing the same cDNA. Approximately 25% of the mass of the mature chitinase in molting fluid is due to carbohydrate, and the predicted mass of the protein portion of the 85 kDa enzyme is about 62 kDa (Koga *et al.*, 1983; Gopalakrishnan *et al.*, 1995). The N-terminus of the 46 kDa enzyme has the same sequence as that of the mature enzyme, and there is no evidence for an internal cleavage in the protein as shown by the detection of only a single N-terminal amino acid sequence. Therefore, the 46 kDa recombinant chitinase found in transgenic plants must represent a truncated molecule that is missing a C-terminal stretch of about 16 kDa.

Two independent methods for determination of the molecular mass of the truncated protein have yielded values of approximately 46.1 kDa. Based on these determinations, we predict that the 46 kDa protein has a total of 407 amino acids (Kramer *et al.*, 1993). Even though the C-terminal portion and glycosylation are not essential for the enzymatic activity, the difference in specific activity between the full length form and the truncated

TABLE 1. Effect of recombinant insect chitinase on growth and mortality of the merchant grain beetle, *Oryzaephilus mercator*

Treatment	Larval weight (mg)†	Larval mortality (%)
Control	0.49±0.02	7
46 kDa chitinase*	—	100‡

*Enzyme (46 kDa chitinase) added to raw wheat germ diet at ~2% level.
†Weight of larvae after 15 days. Data are the mean values ± SE (n=14).
‡All seven larvae were dead 6 days after egg hatch and were too small for weighing.

form from tobacco is so large that there must be a beneficial effect of the missing part of the protein on the enzymatic activity.

The finding that the 46 kDa protein has the same N-terminus as the mature protein indicates that the processing of the leader peptide in both insect and plant cells is identical. The 407 amino acid stretch from the mature N-terminus contains the two highly conserved sequences found in several chitinases, including one that is believed to be part of the catalytic site (Watanabe *et al.*, 1993, 1994). The 46 kDa protein lacks the serine/threonine-rich and cysteine-rich sequences found near the C-terminus. The serine/threonine-rich region bears similarity to sequences in mucins and lectins, which are implicated in the binding with carbohydrate moieties of cell surface glycoproteins (Strous and Dekker, 1992). Apparently, the 85 kDa native enzyme found in insect molting fluid has extensive O-glycosylation of the Ser/Thr domain. Since the 46 kDa protein is enzymatically active, chitinolytic activity is not dependent on either glycosylation or the C-terminal portion of the native protein. The activity of another chitinase from the parasitic nematode *Acanthocheilonema viteae* is also not dependent upon glycosylation (Adam *et al.*, 1996). The carboxyl-terminal cysteine residues that are conserved in several insect chitinases and other proteins also are not essential for chitinase activity (Fuhrman, 1995; Choi *et al.*, 1996; Blaxter, 1995).

The properties of the 46 kDa chitinase are consistent with an organization of insect chitinase into multiple domains with distinctive functions. The 46 kDa protein is enzymatically active with a fourfold lower specific activity and has a slightly altered pH-activity profile than the 85 kDa protein. Whether the lower specific activity is due to a higher K_m or lower V_{max} or both was not determined. The full-length insect chitinase possesses a C-terminal domain similar to yeast chitinase. The C-terminal region of a yeast chitinase has been shown to be important for chitin-binding (Kuranda and Robbins, 1991), but it is not clear whether this chitin-binding site increases the processivity of the yeast enzyme. It will be interesting to compare the chitin-binding properties of the 46 and 85 kDa proteins from *M. sexta*.

The 46 kDa chitinase accumulates in an intracellular location rather than in the apoplastic space, as might be expected from the fact that the 85 kDa enzyme is secreted into the molting fluid of the insect. This localization is surprising since the transit signal peptide appears to be cleaved at precisely the same position of the preprotein both in the plant and the insect as inferred from the N-terminal sequence analyses, indicating similar processing pathways in both systems. However, in transgenic plants, the targeting has been altered from an extracellular to an intracellular location. The absence of the C-terminal region might have affected the targeting of the 46 kDa chitinase in tobacco plants.

It is worth noting that the truncated protein is missing the serine/threonine-rich mucin-like domain that has been implicated in cell adhesive or anti-adhesion properties for this class of proteins (Strous and Dekker, 1992). A similar situation regarding altered targeting has been reported in the case of transgenic tobacco plants expressing hen egg white lysozyme (HEWL); those plants accumulated the enzyme intracellularly even though HEWL is normally a secreted protein (Trudel *et al.*, 1995).

Although the recombinant chitinase is expressed in all of the plant tissues tested, the level of accumulation is highest in the middle leaves and flowers and lowest in the roots and stems. These findings are similar to those of Gatehouse *et al.* (1992), who reported variable levels of expression of genes driven by the CaMV 35S promoter in different cell types, and those of Trudel *et al.* (1995), who found that expression of the HEWL gene under the control of this promoter was highest in leaves, stems and roots. An age-dependent variation in the expression of the chitinase gene in leaves was also observed. The highest level of accumulation was found in leaves from the middle portion of the plant. The lowest levels of the 46 kDa chitinase in younger and older leaves can be attributed to either a slower rate of synthesis and/or a faster rate of degradation.

The level of expression of the 46 kDa chitinase in the transgenic tobacco plants is rather low, reaching only 0.02% of the total protein (Ding, 1995). Northern blot analysis has revealed that mRNA levels are reasonably high. Furthermore, probes corresponding to the 5′ and 3′ regions of the chitinase cDNA clone detect transcripts of the same size (unpublished data). Therefore, the lower efficiency of accumulation and the shorter size of the transgenic chitinase protein in transgenic tobacco cannot be ascribed to inadequate or incomplete transcription. More likely, the translation of the heterologous insect mRNA in plant cells is an inefficient process and prone to premature termination or post-translational processing. Modification of the codons for the insect chitinase gene might be required to enhance translational efficiency, as has been done in the case of mRNA for *Bacillus thuringiensis* toxin.

Although the level of expression is low, an adequate amount of the 46 kDa protein was purified from the transgenic tobacco plants for use in a bioassay with the merchant grain beetle. The 100% mortality of larvae resulting from a 2% level in the diet suggests that the 46 kDa enzyme has potential for use as an insecticidal protein in transgenic plants. In crude extracts of tobacco, the recombinant chitinase represents only 8% of the total activity. How this minor contribution of chitinase activity by the transgene enhances the insecticidal activity of the engineered plants is unknown (Ding, 1995). Apparently, insects are immune to the action of plant chitinases but are quite susceptible to low levels of *M. sexta* chitinase. We are currently in the process of obtaining additional 46 and 85 kDa chitinases as well as endogenous tobacco chitinases for testing against a variety of insects and for determining LD_{50} values. Those experiments should provide more information about the potential of the *M. sexta*

chitinase to act as a bioinsecticide for insect control using transgenic plants and biological control agents.

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